Dissection of *UME6* Point and Truncation Mutants Effects on *C. albicans* Filamentous Growth

An Honors Thesis (HONR 499)

by

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Abstract

Candida albicans is a fungal pathogen that is dangerous when it enters the bloodstream and as it can cause Candidiasis. Candidiasis lethality in immunocompromised individuals and *C. albicans* antifungal drug resistance has made it critical that we understand *C. albicans* biology at a molecular level. Ume6 is a transcription factor important for *C. albicans* filamentous growth and virulence. For this project, I used CRISPR-mediated mutagenesis to create and characterize mutants that substituted two conserved positively charged lysines with two negatively charged glutamates and truncated Ume6 upstream of the Zn^{2+} finger DNA binding and C-terminal domains. Using filamentation assays, I demonstrated that there is decreased filamentous growth with truncation and glutamate substitution mutations upstream of the Ume6 Zn^{2+} finger DNA binding domain. Using virulence tests with *Galleria mellonella* larvae, I demonstrated that there is decreased mortality with truncation and glutamate substitution mutations upstream of the Ume6 Zn^{2+} finger DNA binding domain. Our data supported the hypothesis that the Ume6 Zn^{2+} finger DNA binding domain is important for filamentous growth regulation and virulence.

Acknowledgements

I would like to thank my thesis advisor Dr. Douglas Bernstein for his support and guidance throughout this project. His help and encouragement during my time at Ball State University has positively impacted my abilities as a student and a researcher. However, this is not the only aid I have received in my time here. I would like to thank my lab colleagues Ben, Ethan, and Elena for encouraging and helping me through this difficult project.

Process Analysis Statement

The goal of this project was to understand the importance of the Ume6 Zn^{2+} finger DNA binding and C-terminal domains in *Candida albicans* filamentous growth. This was done by creating and characterizing *C. albicans* strains with altered residues upstream of the Zn^{2+} finger DNA binding domain and C-terminal domain. I performed filamentation assays and virulence tests with *Galleria mellonella* larvae to determine the effects of glutamate substitution and truncations upstream of Ume6 Zn^{2+} finger DNA binding domain and C-terminal domain had on filamentous growth.

My time as a Research Assistant in the Bernstein Lab has taught me many of the technical skills necessary in research, including how to design and carry out my own experiments. I have applied CRISPR-mediated genome editing for the past two years with the last year focusing on creating and characterizing mutants in *UME6*. I have been trained in primer design, DNA recombination, gel electrophoresis, competent cell transformations, *C. albicans* transformations, colony PCR, restriction digestion screening, filamentation assay plating, and *G. mellonella* injections as a Research Assistant in the Bernstein lab, and I used all these skills to carry out my thesis project. For example, I used SnapGene software and our lab databases to design repair template primers to create and insert the mutations into *UME6*. The repair templates were designed with restriction sites for screening. Failure to design the primers correctly can result in ineffective restriction enzyme screening and inability to identify the mutation.

Throughout my application of CRISPR with *C. albicans*, I learned that failure is often more prevalent than success. It takes weeks to perform a *C. albicans*

transformation, confirm mutants, and characterize strain phenotypes. If a problem were identified in any of these procedures, weeks of work would be lost. These problems provided opportunities to learn how to troubleshoot and tackle unexpected outcomes. For example, I was having trouble confirming the *UME6EE* mutant. After discussion with Dr. Bernstein, an agarose gel with purified and unpurified colony PCR was ran to look for any difference in DNA amplification in each sample. I used gel electrophoresis to determine that there was no DNA in the purified colony PCR sample. The protocol for the project was modified to remove the purification step and a successful *UME6EE* mutant was confirmed.

Research is a social experience with discussion and presentation of data at various conferences and presentations. I presented my thesis work at the Midwest Yeast Conference at Northwestern University in Chicago, Illinois during the fall semester of my senior year. That spring, I presented at the 2019 Student Symposium at Ball State University in Muncie, Indiana, and the Undergraduate Research Conference at Butler University in Indianapolis, Indiana. Through these conferences, I learned how to effectively communicate and discuss my research to a variety of audiences. For example, a student more versed in computer science than biology came up to me and asked about my work. I explained my work to him in a way that reflected understanding and generated relevant questions, and he seemed to understand the project's methods and results. It is very useful in this field to understand how to discuss research with a variety of audiences.

Dissection of *UME6* Point and Truncation Mutants Effects on *C. albicans* Filamentous Growth

Introduction

Candida albicans is an opportunistic human fungal pathogen, but its growth is controlled through a healthy immune response [1]. Immunocompromised individuals have a weakened immune response to the pathogen, and the infection can reach the bloodstream. The infection spreads to other solid tissues causing a lethal systemic infection called Candidiasis [2]. With over four million global cases of infection reported annually, there is an increasing concern with Candidiasis infections [3]. Hyphal and yeast growth forms are required for *C. albicans* virulence (Figure. 1) [2]. *C. albicans* infection treatments have become less effective due to the pathogen's antifungal drug resistance [4]. *C. albicans* various antifungal drug resistance mechanisms limit antifungal treatments' effectiveness [5]. These mechanisms reduce an antifungal drug's intracellular accumulation and decrease a drug's affinity or ability to be processed [6]. The exact mechanism of drug resistance used will depend upon the treatment compound, frequency, and dose concentration [7]. To identify better antifungal drug targets and understand filamentous growth, *C. albicans* biology needs to be better understood on a molecular level.

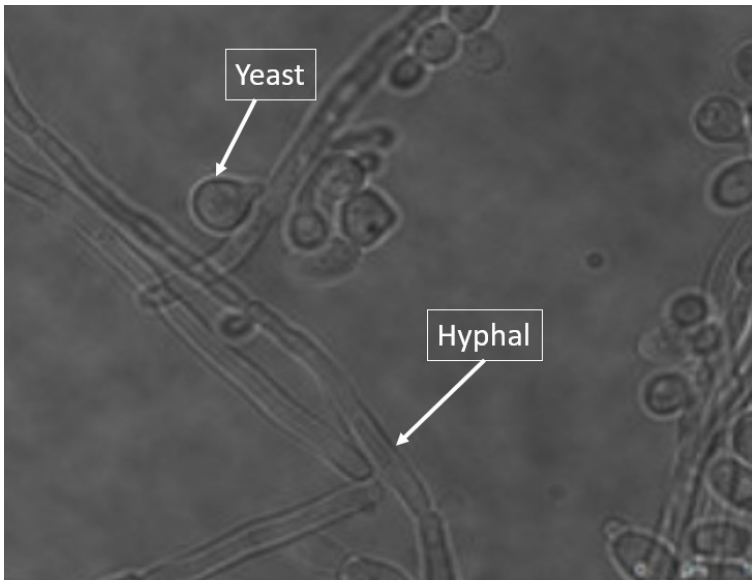

Figure 1. *C. albicans* grows in two forms: yeast and hyphal**.**

C. albicans filamentous growth can be induced by a variety of environmental conditions such as alkaline pH and high temperature [8]. Once hyphal growth forms have developed, the infection is able to spread [9]. If *C. albicans* enters the bloodstream, the cells will spread and grow in other organs, resulting in Candidiasis [10]. Filamentous growth also leads to biofilm formation, which can be detrimental to tissue health and difficult to treat [9]. Research into filamentous growth has been focused on genes involved in regulation of filamentous growth progression [11].

One procedure used to better understand protein function is site-directed mutagenesis. Site-directed mutagenesis is a process that replaces a wild type DNA sequence with a different sequence resulting in an altered version of the protein. Alterations in the phenotype are assessed to determine the protein's function in a process [12]. This process has proven difficult in *C. albicans* for multiple reasons. *C. albicans* has a diploid genome, which means both alleles of a gene need to be modified to study the protein's function [13]. Site-directed mutagenesis commonly uses plasmid

vectors to transform gene mutations into cells [14]. Since *C. albicans* lacks natural plasmids, plasmids are not readily maintained by the cells [11] and lower rates of successful recombination and transformation occur [15].

To get around challenges associated with *C. albicans* transformation, CRISPR (Clustered Regularly-Interspaced Short Palindromic Repeats)-mediated mutagenesis is used [16]. The CRISPR system is used in bacteria and archaea as an immune response against viruses [17]. When bacteria and archaea with CRISPR are attacked by a virus, they retain part of the virus's DNA and incorporate it into their genome [18]. When the surviving cells are attacked later by the same virus, they replicate a guide RNA sequence from this incorporated viral DNA. A catalytic enzyme called Cas9 binds to this guide RNA, and the guide RNA helps facilitate binding to the invading viral DNA. The bound Cas9 cleaves the DNA and disables it [19].

Researchers have developed ways to leverage CRISPR technology in various multicellular organisms to edit DNA (Figure 2). CRISPR uses the Cas9 enzyme bound to a guide RNA to facilitate binding to the homologous DNA and protospacer adjacent motif (PAM) site in the target gene. The PAM site serves as the Cas9 targeting site for CRISPR [20]. Once bound downstream of the PAM site, Cas9 cleaves the gene and leaves the gene inoperable if not repaired. CRISPR-mediated recombination takes advantage of this break by using homologous recombination to modify the gene with a repair template containing a mutation of interest [21].

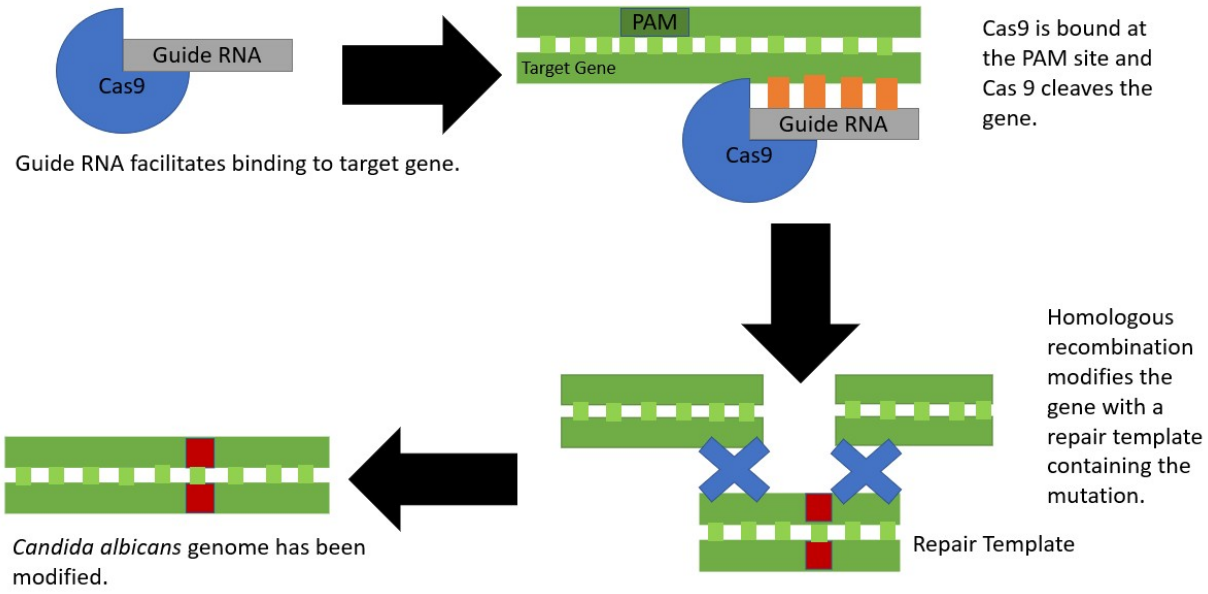

Figure 2. Model of CRISPR-mediated genome editing used to modify *C. albicans* genome. The blue X's indicate homologous recombination. The mutation is indicated in red.

One of the genes associated with filamentous growth that we wanted to apply this procedure to is *UME6.* Ume6 is a transcription factor important for filamentous growth and virulence. It controls filamentous growth by regulating expression of genes that promote hyphal elongation, germ tube formation, and virulence [22]. Ume6 contains a Zn^{2+} finger DNA binding domain between the N-terminal and C-terminal domains (Figure 3A) [23]. The goal of this project was to determine if the Zn^{2+} finger DNA binding and C-terminal domains are required for filamentous growth regulation. This was done by altering residues upstream of the Zn^{2+} finger DNA binding and C-terminal domains (Figures 3B, 3C, and 3D).

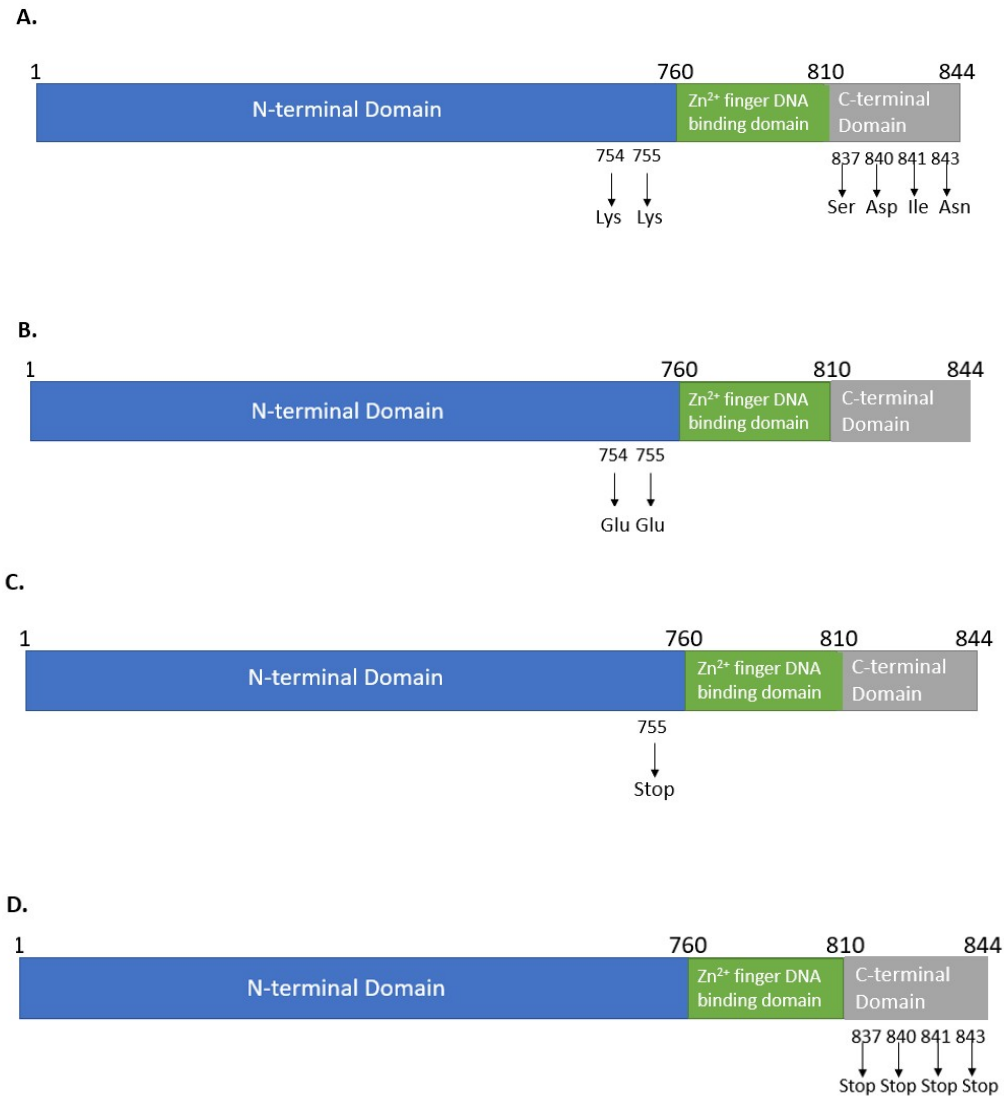

Figure 3. Cartoon of the Ume6 protein domains. The sites indicated show where in the amino acid sequence each mutation was located. **A.** wild type Ume6. **B.** *UME6EE* mutation. **C.** *UME6STOP* mutation. **D.** C-terminal domain truncations.

In summary, *C. albicans* is an opportunistic human fungal pathogen that has the potential of developing into a lethal systemic infection called Candidiasis. The immune response of a healthy individual is strong enough to prevent bloodstream infection and Candidiasis, but an immunocompromised individual is more susceptible. Treatment complications arise with *C. albicans* antifungal drug resistance. These reasons have led to investigation of *C. albicans* biology on a molecular level. Site-directed mutagenesis

inserts a mutation into a gene to understand the gene's function. This process has proven difficult in *C. albicans* because of the yeast's diploid genome and lack of natural plasmids. To get around this, we use CRISPR mutagenesis to mutate *C. albicans* genes by using Cas9 to cleave a target gene and using homologous recombination to modify the gene. We have modified Ume6, a transcription factor important for filamentation, using this process to investigate if the Zn^{2+} finger DNA binding and C-terminal domains are required for filamentous growth.

Methods

C. albicans Cloning and Sequencing

The *UME6* guide primers (Table 1) were subcloned into pv1093 [21]. Repair templates for all mutations were made using PCR with repair template primers listed in (Table 1). Repair templates were purified with a Zymo PCR Purification Kit. *C. albicans* was transformed using a lithium acetate transformation protocol and plated on YPD (yeast extract peptone dextrose) media with uridine and 2X nourseothricin (NAT). The transformants were grown at 30 °C [24]. Transformants from these plates were streaked for singles on YPD with uridine and 1X NAT and grown at 30 °C. Colony PCR was performed on the single colonies using the primers listed in (Table 1). The colony PCRs were resolved on 2% agarose gel treated with ethidium bromide. The colony PCRs were screened using restriction digestion with either PacI or BseRI. Restriction digests were resolved on a 2% agarose gel treated with ethidium bromide. Successfully digested PCRs were sequenced to verify correct genome editing. Correct transformants were stored at -80 ºC for future use.

Oligo Name	Sequence	Restriction Site
Ume6 Guide 2 fr	***ATTTG***tacttctacttctaatccaa***G***	XXX
Ume6 Guide 2 rv	***AAAAC***ttggattagaagtagaagta***C***	XXX
Ume6 rp 2_TAA fr	aatggcactaacaccaatactgattctacttctacttctaatccaatggt**TTAATTAA**aa	Pac1
Ume2 rp 2_TAA rv	tccttttttagatctaggtaataatcttcttcttgtatgttt**TTAATTAA**accattggat	Pac1
Ume6 rp 2_Glu fr	atggcactaacaccaatactgattctacttctacttctaatccaatggtg**GAGGAG**aaac	BseRI
Ume6 rp 2_Glu rv	catccttttttagatctaggtaataatcttcttcttgtatgttt**CTCCTC**caccattgga	BseRI
Ume6trunc1 Fw	aaaagaaatcaagctaaaacaaaaattctcggaaaaaatcaagtaatgata**TTAATTAA**	Pac1
Ume6trunc1 Rv	ctgttaattcttaattcttaacttagcctaatttcaatcattca**TTAATTAA**tatcatta	Pac1
Ume6TAA-3 RV	gtcaacctgttaattcttaattcttaacttagcctaatttcaatcattg**TTAATTAA**tat	Pac1
Ume6TAA-3 Fw	caagctaaaacaaaaatttctcggaaaaaatcaagtaatgata**TTAATTAA**caatgat	Pac1
Ume6TAA-4 Rv	ctgttaattcttaattcttaacttagcctaatttcaatcattg**TTAATTAA**tcattactt	Pac1
Ume6TAA4 Fw	aaagaaatcaagctaaaacaaaaatttctcggaaaaaatcaagtaatga**TTAATTAA**	Pac1
Ume6trunc7 Fw	aaaagaaatcaagctaaaacaaaaatttctcggaaaaaatcaagtaatg**TTAATTAA**t	Pac1
Ume6trunc7 Rv	ttcttaattcttaacttagcctaatttcaatcaatcattgtta**TTAATTAA**cattacttg	Pac1
Ume6 ch primer 2 fr	ggtcatgatcatgatgatgaaaat	XXX
Ume6 ch primer 2 rv	ctccacaaattggtgtgacttc	XXX
ume6-7-8-4 fr	gtggaggttgtgctaaattt	XXX
ume6-7-8-3 rv	ttgttggaatcttctacggg	XXX

Table 1. Primers designed to create and verify *UME6 C. albicans* mutants. Capitalized, bolded, and italicized sequences indicate ligation sites at sticky ends in the plasmid. Capitalized and bolded sequences indicate restriction sites.

Growth and Filamentation Assays

Wild type, *UME6EE*, and *UME6STOP C. albicans* strains were grown overnight in YPD media at 25 °C. The strains were diluted to OD 0.1. Fourfold serial dilutions were plated on YPD and Spider media plates with a pin replicator and incubated at 30 °C and 37 °C overnight [21].

Galleria mellonella Injections

Healthy *G. mellonella* larvae were randomly assigned into wild type, *UME6STOP*, *UME6EE*, and phosphate buffered saline (PBS) control groups. Larvae were cleaned with ethanol and injected with 10^5 colony-forming unit (cfu). *C. albicans* wild type or mutant cells. PBS was injected into one group of wax worms to serve as a control. The *G. mellonella* larvae were incubated at 37 °C and observed daily for mortality for 7 days.

Results

Colony PCR and Restriction Digestion Screening

Ume6 is comprised of three domains hypothesized to be involved with filamentous growth regulation. This project focused on understanding the importance of the Ume6 Zn^{2+} finger DNA binding and C-terminal domains. Conserved positively-charged lysines upstream of the Ume6 Zn^{2+} finger DNA binding domain were substituted with negatively-charged glutamates that altered Ume6 function because of the amino acids' different charges [25]. Truncations upstream of the Ume6 Zn^{2+} finger DNA binding and C-terminal domains were performed to determine if the Ume6 Zn^{2+} finger DNA binding domain is necessary for filamentous growth. Repair templates were

designed to modify *UME6* upstream of the Zn^{2+} finger DNA binding and C-terminal domains. Purified repair template and plasmid containing the *UME6* guides were transformed into *C. albicans*. All transformants were streaked for singles and colony PCR was performed. Restriction enzyme digestion was performed on the colony PCR to screen for the correct incorporation of repair templates (Figure 4). From this, transformants were identified that successfully incorporated *UME6STOP* and *UME6EE* repair templates (Table 2) (Figures 3B and 3C).

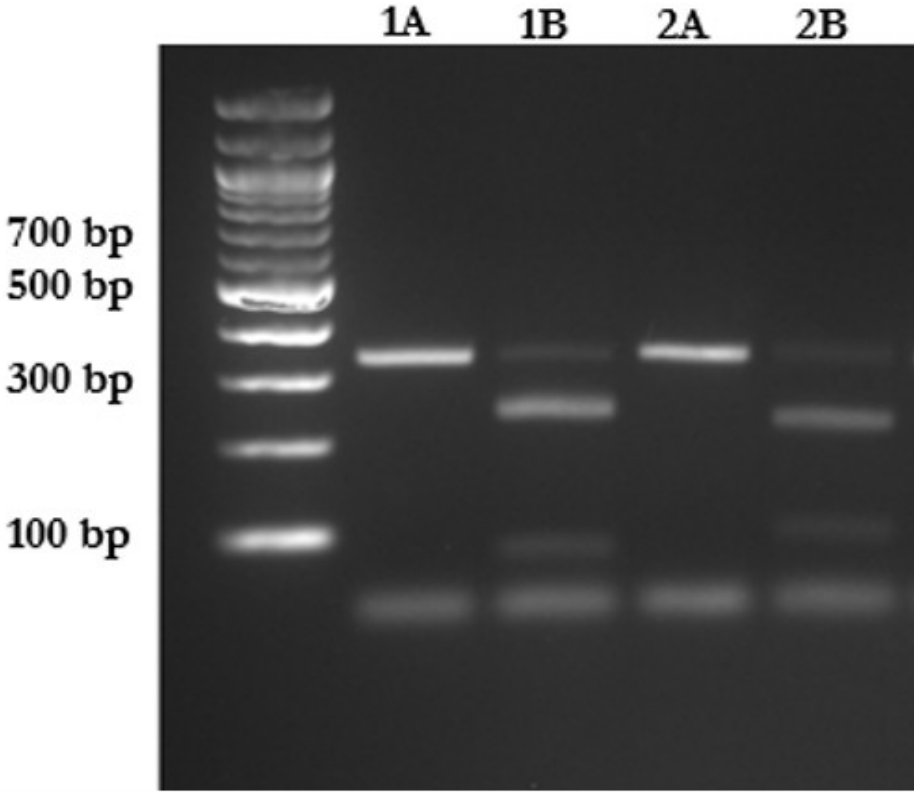

Figure 4. Colony PCR and restriction enzyme digest of *C. albicans UME6* transformants**.** Columns 1A and 1B show colony PCR and digest of *UME6STOP* with Pac1. Columns 2A and 2B show PCR and subsequent digestion of *UME6EE* with BseR1.

Name	**Genotype**	**Reference in Text**
SC5314	*UME6:UME6*	wild type
DAB898	*ume6-LYS754E LYS755E:ume6-LYS754E LYS755E*	*UME6EE*
DAB89	*ume6-LYS754stop:ume6-LYS754stop*	*UME6STOP*

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Table 2. *C. albicans* strains used in the project.

Filamentation Assays

Filamentation assays were performed to determine the effect of glutamate substitution and truncation upstream of the Ume6 Zn^{2+} finger DNA binding domain on filamentous growth. Wild type*, UME6EE,* and *UME6STOP* cultures were plated on Spider media and grown at 30 ºC and 37 ºC (Figure 5). *UME6EE* had decreased filamentous growth compared to wild type at 30 ºC and 37 ºC. *UME6STOP* did not filament at 30 °C and 37 ºC. Wrinkled colony morphology was less pronounced in *UME6EE* at 30 ºC and 37 ºC compared to wild type. Wrinkled colony morphology was less pronounced in *UME6STOP* compared to wild type at 37 ºC. *UME6STOP* had smooth colony morphology at 30 ºC. Using filamentation assays, we found truncations and glutamate substitution upstream of the Ume6 Zn^{2+} finger DNA binding domain decreased filamentous growth in *C. albicans*.

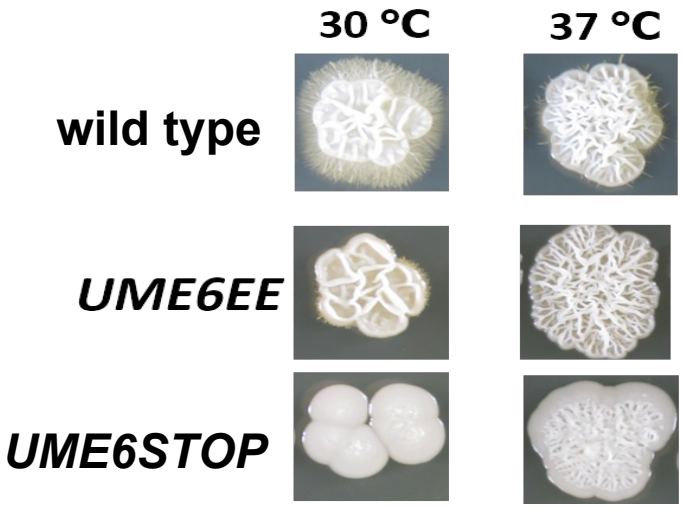

Figure 5. Filamentation assay of wild type*, UME6EE,* and *UME6STOP* at 30 ºC and 37 ºC on Spider media.

Virulence Tests

Virulence tests were performed to determine the Ume6 strains virulence in *G. mellonella* larvae. *G. mellonella* larvae were used since their innate immune systems are like those in mammals. *G. mellonella* larvae were injected with a PBS control, wild type*, UME6STOP,* or *UME6EE* and observed daily for one week (Figure 6). Only 20% of the *G. mellonella* larvae treated with the wild type strain survived. 40% of the *UME6EE*-treated *G. mellonella* larvae survived the infection. *UME6STOP*-treated *G. mellonella* larvae had approximately 80% of the larvae survive. The PBS control group had 95% larvae survival. Overall mortality was decreased in *UME6STOP*- and *UME6EE*-treated larvae compared to wild type. The *UME6STOP* larvae exhibited the lowest mortality. Using the virulence tests, it was determined that glutamate substitution and truncation upstream of the Ume6 Zn^{2+} finger DNA binding domain decrease virulence.

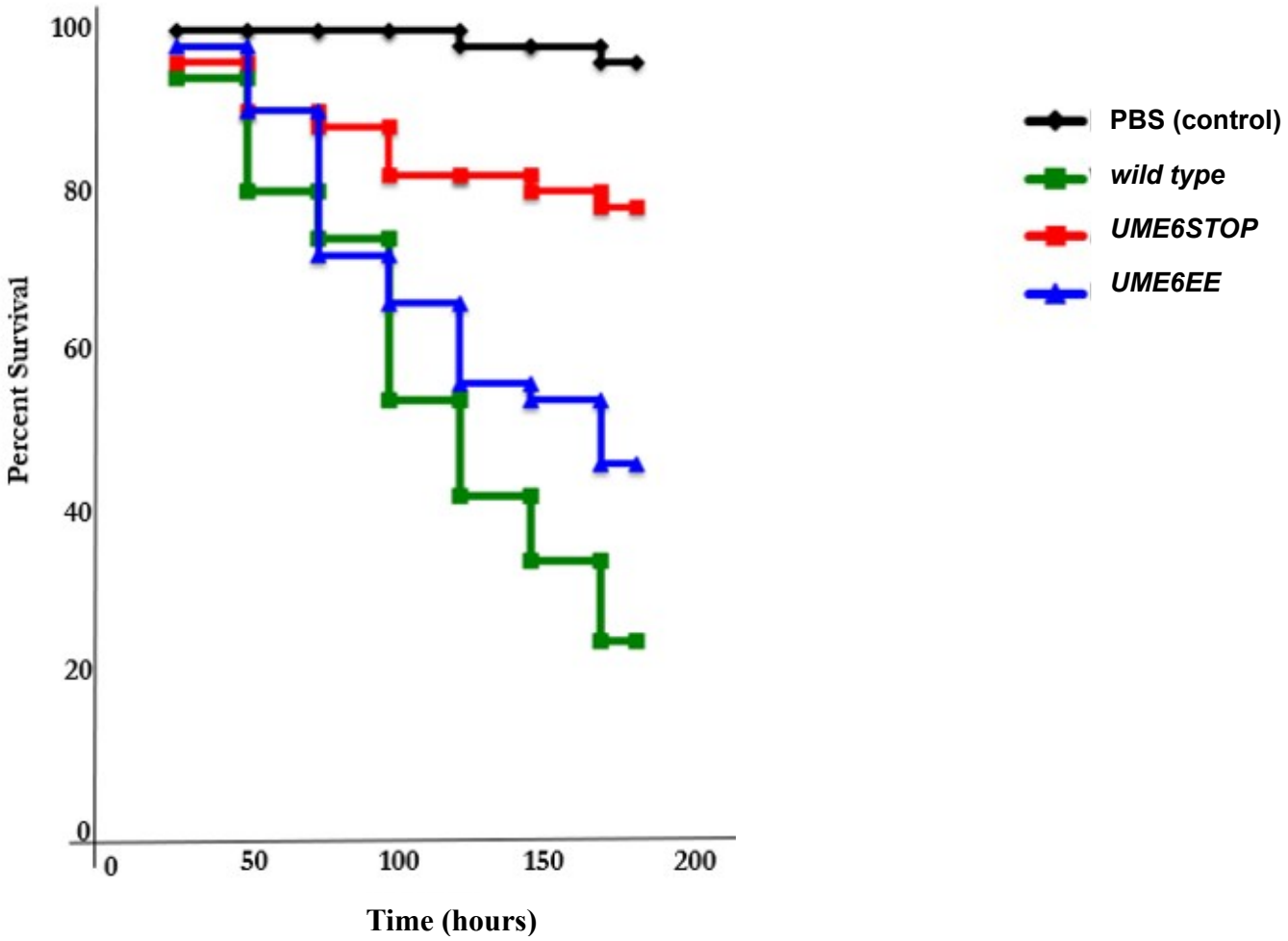

Figure 6. Survival of *G. mellonella* larvae injected with wild type, *UME6STOP* and *UME6EE* strains overtime.

Ume6 C-Terminal Domain Truncations

The effects on filamentous growth and virulence observed our truncation upstream of Ume6 Zn^{2+} finger DNA binding domain prompted investigation of the Ume6 C-terminal domain role in filamentous growth. Guide RNAs and repair templates truncating one, three, four, and seven amino acids from the Ume6 C-terminal domain were designed and transformed into *C. albicans* (Figure 3D). We did not recover any Ume6 C-terminal domain truncations in *C. albicans*.

Discussion

Glutamate substitution and truncation upstream of the Ume6 Zn^{2+} finger DNA binding domain were generated to determine involvement of the Ume6 Zn^{2+} finger DNA binding domain in filamentous growth and virulence. Substitution of two glutamates for two conserved lysine residues upstream of the Ume6 Zn^{2+} finger DNA binding domain reduced filamentous growth and virulence compared to wild type *C. albicans* (Figure 5 and 6). Truncation upstream of Ume6 Zn^{2+} finger DNA binding domain eliminated filamentous growth (Figure 5) and demonstrated a larger decrease in virulence than *UME6EE* when compared to wild type (Figure 6). Our data supports the hypothesis that the Ume6 Zn^{2+} finger DNA binding domain is important for promoting filamentous growth. Zn^{2+} finger proteins are transcriptional regulators and other *C. albicans* transcriptional regulators involved with filamentous growth regulation, like FGR17 and FGR27, contain Zn^{2+} finger DNA binding domains [26]. Other *C. albicans* transcriptional regulators, like those in the NDT80 family that also contain Zn^{2+} finger DNA binding domains, regulate other processes such as stress response [27]. Thus, transcriptional regulators with Zn^{2+} finger DNA binding domains are not limited to filamentous growth regulation.

The Ume6 Zn^{2+} finger DNA binding domain is important for filamentous growth regulation. We did not know if both the Ume6 Zn^{2+} finger DNA binding and C-terminal domains were required for filamentous growth. I attempted to truncate the Ume6 C-terminal domain to see if the Zn^{2+} finger DNA binding domain is sufficient for filamentous growth regulation. I successfully cloned *UME6* guides and made repair templates for different Ume6 C-terminal domain truncations. The truncation mutations were not present in the transformed *C. albicans*. Multiple potential reasons exist for why the

Ume6 C-terminal domain truncations were not recovered. Previous work in the lab introducing stop codons into *UME6* has found that a lack of repair template homology can inhibit successful genome editing. Homologous recombination efficiency can be increased by increasing the size of the homologous region of the repair template and increasing the amount of repair template added to the transformation. Increasing repair template homology could be done by generating primers that increase homology at either end of the repair template. To do this I would have to make longer repair templates. This, however, can be cost prohibitive as longer primers cost more to produce.

Another possible explanation for why the Ume6 C-terminal domain truncations were not present in *C. albicans* is that truncation of the Ume6 C-terminal domain is lethal. One way this could occur is if the C-terminal domain truncation caused a dominant negative effect by interaction with essential protein complexes. It has been shown that dominant-negative mutations that truncate a protein and leave only a DNA binding domain can turn the protein into a transcriptional inhibitor [28]. Furthermore, Ume6 interacts with proteins, such as Ras G family proteins, that are involved with multiple signaling pathways [29]. If a dominant-negative effect occurred in a Ume6 C-terminal truncation mutant, this inhibition could shut down some of these essential pathways. Another way Ume6 could be lethal is if the lack of C-terminal domain caused random Zn^{2+} finger DNA binding that is toxic. Alterations to gene expression caused by rogue Zn^{2+} finger binding activity can cause gene reprogramming and unintentional transcriptional regulation [30]. For example, this occurs with the testis zinc finger protein (TZFP) N-terminal domain that regulates generation of virus-specific natural cells in

mice. Altered TZFP residues, such as Phe44 or Ser45, alter DNA interactions. This results in abnormal virus-specific natural killer cell production [31].

It would be interesting to continue investigation of Ume6 C-terminal domain truncations. This project has given supporting evidence that the Zn^{2+} finger binding domain and the Ume6 C-terminal domain regulate filamentous growth. It has not verified if the Ume6 Zn^{2+} finger binding domain is sufficient for filamentous growth regulation. Further work needs to be done to verify Ume6 C-terminal domain involvement in filamentous growth regulation to better understand the interaction mechanisms between Ume6 and the cellular components necessary for filamentous growth. Other possible Ume6 C-terminal domain mutations potential amino acid substitutions in the C-terminal domain and additional C-terminal domain truncations could be made. Glutamate and alanine substitutions and truncations have been successfully incorporated into Ume6. Designing mutations upstream of amino acids targeted with the Ume6 C-terminal domain truncations attempted in this project could be challenging as no PAM sites in exist in this portion of *UME6*. One way around this using the new CRISPR system Cpf1. Cpf1 utilizes the PAM site TTTN instead of NGG [32]. Cpf1 PAM sites are more abundant in Ume6 than Cas9 PAM sites and use of the Cpf1 PAM sites would help get around the lack of Cas9 PAM sites.

In the future, more work should be done applying CRISPR mutagenesis to other *C. albicans* genes to better understand their involvements with filamentous growth. Filamentous growth in yeast occurs when environmental circumstances trigger signaling pathways that cause cell elongation, reorganized polarity, and increased cell-to-cell adhesion [33]. Current lab members are applying CRISPR mutagenesis to *UME7,*

another transcription factor, and various pseudouridine synthase (PUS) genes. While Ume7 is thought to act as a transcription factor in *C. albicans*, a similar *Saccharomyces cerevisiae* gene, *UME6*, regulates meiotic genes [23]. Meiosis is used for reproduction in *S. cerevisiae*, but *C. albicans* does not perform meiosis [34]. This leads the question as to the role of *UME7* in *C. albicans.*

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